REVIEW ARTICLE

MicroRNAs in Lipid Metabolism and Atherosclerosis

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Abstract

BACKGROUND: MicroRNAs (miRNA) are mediators of post-transcriptional gene expression that likely regulate most biological pathways and networks. The study of miRNAs is a rapidly emerging field; recent findings have revealed a significant role for miRNAs in atherosclerosis and lipoprotein metabolism.

CONTENT: Results from recent studies demonstrated a role for miRNAs in endothelial integrity, macrophage inflammatory response to oxidized low-density lipoprotein, vascular smooth muscle cell proliferation and cholesterol synthesis. These mechanisms are all vital to the initiation and proliferation of atherosclerosis and cardiovascular disease. The importance of miRNAs has recently been recognized in cardiovascular sciences and miRNAs will likely become an integral part of our fundamental comprehension of atherosclerosis and lipoprotein metabolism. The extensive impact of miRNA mediated gene regulation and the relative ease of *in vivo* applicable modifications highlight the enormous potential of miRNA-based therapeutics in cardiovascular diseases.

SUMMARY: miRNA studies in the field of lipid metabolism and atherosclerosis are in their infancy, and thus there is tremendous opportunity for discovery in this understudied area. The ability to target miRNAs *in vivo* through delivery of miRNA-mimics to enhance miRNA function, or anti-miRNAs which inhibit miRNAs, has opened new avenues for the development of therapeutics for dyslipidemias and atherosclerosis, offers a unique approach to treating disease by modulating entire biological pathways. These exciting findings support the development of miRNA antagonists as potential therapeutics for the treatment of dyslipidaemia, atherosclerosis and related metabolic diseases.

Abstrak

LATAR BELAKANG: *MicroRNAs* (miRNA) merupakan mediator ekspresi gen pasca transkripsi yang mengatur hampir seluruh jalur dan jaringan biologis. Penelitian mengenai miRNA berkembang pesat; penemuan terbaru menunjukkan peran signifikan miRNA pada aterosklerosis dan metabolisme lipoprotein.

ISI: Hasil penelitian terkini menunjukkan peran miRNA pada integritas endotel, respon inflamasi makrofag terhadap *oxidized low-density lipoprotein*, proliferasi sel otot polos vaskular, dan sintesis kolesterol. Semua mekanisme tersebut berperan penting pada inisiasi dan proliferasi aterosklerosis dan penyakit kardiovaskular. Kesadaran akan pentingnya miRNA bagi ilmu kardiovaskular tampaknya akan menjadikan miRNA sebagai bagian tak terpisahkan dari perkembangan fundamental aterosklerosis dan metabolisme lipoprotein. Efek miRNA yang luas dalam memediasi regulasi gen serta relatif mudahnya modifikasi pada aplikasi *in vivo* menonjolkan potensi besar terapi berbasis miRNA pada penyakit kardiovaskular.

RINGKASAN: Penelitian mengenai miRNA pada metabolisme lemak dan aterosklerosis masih berada pada tahap dini, sehingga kesempatan untuk penemuan-penemuan di bidang ini masih terbuka luas. Kemampuan menjadikan miRNA sebagai sasaran terapi secara *in vivo* melalui pemberian miRNA-*mimics* (tiruan miRNA) yang dapat meningkatkan fungsi miRNA, atau anti-miRNA yang dapat menghambat miRNA, telah membuka kesempatan baru perkembangan terapi dislipidemi dan aterosklerosis, menawarkan pendekatan yang unik untuk mengelola penyakit melalui modulasi jalur biologis secara keseluruhan. Penemuan yang menarik ini mendukung perkembangan antagonis miRNA sebagai terapi potensial untuk pengobatan dislipidemia, aterosklerosis dan penyakit metabolisme.

KEYWORDS: atherosclerosis, lipoprotein, HDL, miRNA

KATA KUNCI: aterosklerosis, lipoprotein, HDL, miRNA

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Introduction

Mendelian randomization studies have shown that certain single-nucleotide polymorphisms that raise plasma high-density lipoprotein cholesterol (HDL-C) levels do not lower the risk of myocardial infarction, challenging the concept that HDL is atheroprotective.(1) Compounding these findings, several clinical trials of HDL-C-raising therapeutics, including niacin and inhibitors of cholesterol ester transfer protein (CETP), have failed to show benefit.(2,3) These studies have begun to cast doubt on HDL's atheroprotective functions and spurred further investigations of the molecular mechanisms regulating levels of plasma HDL and its function. One area of rapid growth in this regard has been the discovery of microRNAs (miRNAs) as potent regulators of the gene pathways controlling HDL genesis, cholesterol efflux, and reverse cholesterol transport (RCT).

Over 1,500 human miRNAs have been curated in miRBase (miRBase.org), and each cell type typically contains a specific subset of approximately 150-300 miRNAs. One miRNA can target and regulate potentially hundreds of genes (mRNAs) through seed-based targeting, and one gene (mRNA) can be under negative repression of more than one miRNA. The nature of miRNA gene repression is, therefore, complex and highly interconnected with both direct and indirect effects. Nonetheless, miRNA networks modulate many aspects of cellular homeostasis and physiology and thus are a promising new area of investigation.(4)

For the past decade, the importance of noncoding RNA as regulators of pre-, co-, and post-transcriptional gene expression has emerged.(5) Of the various classes of noncoding RNAs, miRNAs are currently the most widely studied and have established roles in regulating a myriad of biological processes.(6) Although miRNAs likely play a role in most homeostatic pathways, they also appear to be powerful regulators in the development of various diseases. Dysregulation or altered miRNA expression/function has been implicated in diabetes (7), cardiovascular disease (8-12), and numerous types of cancers. The role of miRNAs as biomarkers has also gained significant attention in both academic research labs and *in vitro* diagnostic test companies. miRNAs are stable in plasma and differential plasma miRNA profiles have been described for many diseases, including fatty liver (13), atherosclerosis (14-16), and cancer (17-21). Circulating miRNAs have enormous potential as novel disease biomarkers; however, circulating extracellular miRNAs may also be biologically active.

Most of the novel miRNA-mediated regulatory mechanisms associated with atherosclerosis and lipoprotein metabolism to date have first been determined by miRNA profiling, followed by functional testing.(22,23) The discovery of miRNA gene regulatory mechanisms contributing to endothelial integrity, macrophage inflammatory response to atherogenic lipids, vascular smooth muscle-cell proliferation, and cholesterol synthesis. At this time, the applicability and full potential of miRNAs in clinical practice is unknown. Nonetheless, recent advances in miRNA delivery and inhibition hold great promise of a tremendous clinical impact in atherosclerosis and cholesterol regulation.(11)

miRNA

miRNAs are a specific class of noncoding RNA (ncRNA), and are defined as small, 20-22 nucleotide RNA molecules that are processed from a much larger primary transcript. Once processed into their mature form, miRNAs generally bind to complimentary sequences in the 3′ untranslated region (UTR) of specific genes but can also bind to other regions of the gene including the 5′ UTR and the coding region.(24,25) Via mRNA destabilization and/or protein translation inhibition, miRNAs mediate silencing of their bound targets.

miRNAs have diverse expression patterns and might regulate various developmental and physiological processes. Their discovery adds a new dimension to our understanding of complex gene regulatory networks.(26) Two processing events lead to mature miRNA formation in animals. In the first, the nascent miRNA transcripts (pri-miRNA) are processed into ~70-nucleotide precursors (pre-miRNA); in the second event that follows, this precursor is cleaved to generate ~21-25-nucleotide mature miRNAs.(27)

The impact of miRNAs on the proteome indicated that for most interactions of miRNAs act as rheostats to make fine-scale adjustments to protein output.(28) Studies show that a single miRNA can directly downregulate production of hundreds of proteins. In addition to the known effect

on global mRNA levels (29), data strongly indicate that miRNAs translationally repress hundreds of direct target genes (30). miRNAs are involved in a plethora of important biological processes, from embryonic development to homeostasis in adult tissues. Recently, miRNAs have emerged as a class of epigenetic regulators of metabolism and energy homeostasis.(31)

Because of their size, abundance, tissue specificity, and relative stability in plasma, miRNAs hold promise as unique accessible biomarkers to monitor tissue injury. miRNAs have been found to be involved in almost every biological process, from cellular differentiation and proliferation (32,33) to cell death and apoptosis (34,35), from synaptic plasticity (36) to immunity (37) and cardiovascular development (38); in addition, changes in miRNA levels and activity have been linked to human pathologies, including cancer (39) and cardiovascular diseases (40,41). miRNAs can regulate the mRNA levels of their targets (29,42), and pharmacological silencing of miRNAs using antagomirs might therefore lead to the regulation of many mRNAs.(31)

Efficient silencing of miRNA-122 (miR-122) was achieved in primates by three doses of 10 mg/kg locked-nucleic-acid (LNA)-anti-miR, leading to a long-lasting and reversible decrease in total plasma cholesterol without any evidence for LNA-associated toxicities or histopathological changes in the study animals.(43)

Lipid-based Carrier of miRNA and Intercellular Communication

Until recently, the predominant view of intercellular communication was that it was limited to cell-to-cell adhesion conduits (gap junctions) or secreted signals, such as hormones and neurotransmitters. New studies have revealed that plasma-membrane-derived vesicles, namely exosomes and microvesicles, can transfer proteins, mRNAs and miRNAs from donor cells to recipient cells. (44-47) miRNAs are short non-coding regulatory RNAs that modulate biological homeostasis by controlling gene expression through mRNA targeting and translational repression.(28,30,48,49) Circulating miRNAs are a new class of biomarkers and have recently been used for a diverse set of diseases. We now understand that the secretion of miRNAs is a controlled, active, and specific process. miRNAs can be packaged into lipid-based carriers such as exosomes, microparticles, or apoptotic bodies, and have been found on lipoproteins like high- and low-density lipoprotein (HDL and LDL, respectively). Additionally, a significant portion of extracellular miRNAs are found without a lipid carrier, and are protein-bound. While the mechanisms of the selectivity of miRNA packaging remain unclear, researchers are beginning to unravel some of the mysteries surrounding how these tiny RNA molecules make their way out of the cell.(50)

The complexity of miRNA-mediated pathway control has burgeoned since the discovery that miRNAs are found in the extracellular space and constitute a form of cell-cell communication. miRNAs have been found in plasma, urine, and saliva and have recently been shown to be carried on lipoproteins. This has led to the proposal that circulating miRNAs may be useful biomarkers of various diseases, including cardiovascular disease, diabetes, and other forms of dysregulated metabolism.(50)

Given the lability of most RNAs, it is perhaps surprising that circulating miRNAs in plasma are relatively stable and appear to be resistant to plasma ribonucleases (RNase).(51) This protection has been shown to be conferred by their lipid-based carriers that shield the small RNAs from plasma RNases.(51-54) Circulating miRNAs themselves are not inherently resistant to degradation.(55) Once their lipid-based carriers are disrupted, either by the addition of detergents (56) or by sonication (57), they are sensitive to RNase degradation. The most widely studied lipid-based miRNA carriers are exosomes, which are membrane-derived vesicles that originate from endosomal multivesicular bodies.(52,58,59) Exosomes, which are derived from intracellular vesicles of approximately 100nm in diameter, have a similar size range of 40–100nm when released into the interstitial fluid.(60) The external release of exosomes by the fusion of multivesicular endosomes with the plasma membrane is a distinct process that is not shared by other membrane-derived vesicles.

In addition to exosomes, other membrane-bound and lipid-containing vesicles transport miRNA in the extracellular space. Microvesicles are larger than exosomes, typically ranging from 100 nm to 1 μm in size, with most microvesicle preparations comprised of a heterogeneous mixture of particles.(61) Microvesicles are secreted by a number of different cell types, including vascular cells, platelets, and inflammatory cells, and it has been speculated that almost all cell types are capable of secreting microvesicles under specific conditions.(62) Apoptotic bodies are another variety of lipid-encapsulated vesicles known to carry miRNA. These types of vesicles are generally much larger (~500 nm to >2,000 nm) and have a heterogeneous size distribution.(63) As the name suggests, apoptotic bodies are released at the early stages of apoptosis, and contain both a lipid bilayer derived from the plasma membrane and cytoplasmic contents that originate from the parent cell.

In addition to lipid-encapsulated organelles released from cells, lipoproteins have also been shown to associate with miRNA and are arguably the most important lipid carrier in the body. Lipoproteins are comprised of various proteins and lipids and are responsible for the delivery of cholesterol, triacylglycerols, steroids, and fat-soluble vitamins to peripheral tissues from the liver via LDL and removal from peripheral tissues to the liver/digestive system via HDL.(64,65) Due to their inherent solubility and their tendency to trap water-insoluble material in their core, lipoproteins have demonstrated an ability to carry nucleic acids and are often used as gene delivery agents, and it has been demonstrated in a recent study that different RNA structures display varying affinities for phospholipid membranes and this could affect the ability of an RNA molecule to bind to a lipoprotein.(4,46)

Vickers *et al*. (4) were the first to demonstrate that human HDL and LDL have the capability to carry miRNAs in the core of the molecule hidden from the extracellular environment in serum, and most importantly these miRNAs can be transferred to recipient cells and alter target cell gene expression. Importantly, this study revealed, at least in the case of HDL, that the miRNAs carried in these lipoproteins differ between healthy subjects and those with atherosclerotic vessel disease. One of the most abundant miRNAs detected in HDL particles was miR-223 which, intriguingly, was found to be highly enriched in monocytes/macrophages.(46) These data suggest that it may be peripheral cells like macrophages that may be off-loading their miRNA into HDL particles, and not necessarily the cells responsible for HDL biogenesis. HDL-miRNAs could potentially serve as diagnostic markers in much the same way that exosome miRNAs have been used.(66-70). HDL could simply be a depot or carrier for circulating miRNAs and their presence in HDL may not necessarily relate to the function of HDL in atherosclerosis or lipid metabolism. If so, miRNAs in HDL could perhaps be used diagnostically for a wide variety of diseases besides atherosclerosis.(71)

Argonaute proteins (Ago1/2), which act as effector molecules for miRNAs, are detected in cell supernatants and are not necessarily associated with vesicles of any kind (microvesicles or exosomes).(72) The binding of Ago2 to miRNA protects the miRNA from degradation by the abundant amount of RNases found in plasma, and it is believed that Ago2 protein can assist in functional transfer of miRNAs that it carries.(55) Many different proteins and pathways have been linked to exosome export, including the ceramide pathway. Ceramide has been shown to facilitate the formation of endosomal vesicles (73), and the export of specific miRNAs to exosomes (74). Knockdown of neutral

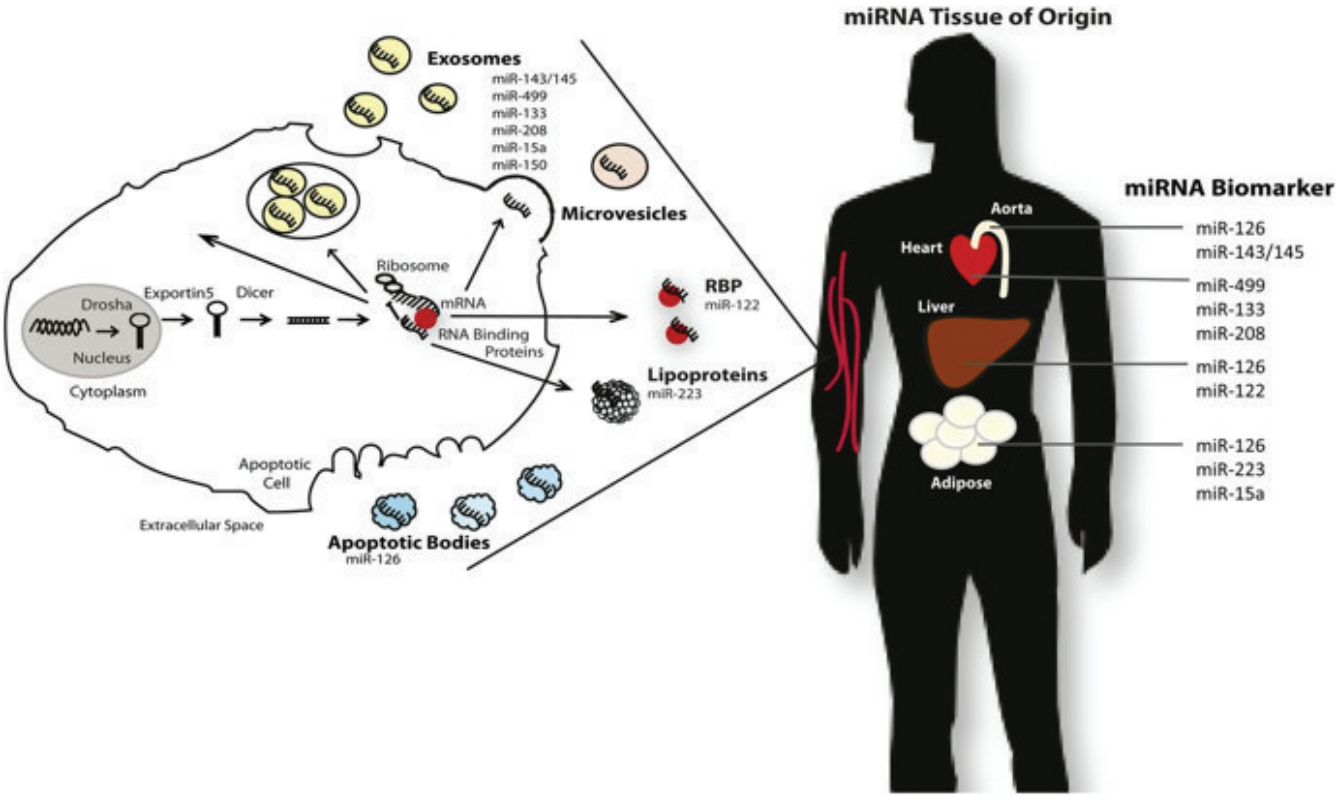

Figure 1. MicroRNAs are secreted into the circulation and are biomarkers for various diseases. (50) (Adapted with permission from American Society for Biochemistry and Molecular Biology).

sphingomyelinase 2 (nSMase2), the key enzyme in ceramide synthesis, with siRNAs leads to a decrease in the cellular export of miR-16 and miR-146a.(52) Less is even known about the cellular uptake of miRNAs than their export, but there are two main hypotheses. For vesicles (microvesicle/exosomes) containing miRNAs, it has been proposed that they are delivered to cells either by a process involving endocytosis (75,76) or by membrane fusion (77,78). Surface or transmembrane proteins on vesicles and/or cell surface receptors likely contribute to the initial recognition of target cells; however, the identity of these cell surface receptors or their ligands is unknown.

Scavenger receptor class B type I (SR-BI) mediates the uptake of miRNAs associated with HDL.(71) Because the selective core uptake of lipids on HDL by SR-BI also bypasses the endosomal–lysosomal pathway (79,80), delivery of miRNAs by this pathway may also lead to less degradation of miRNAs. We are only beginning to understand the physiologic impact of miRNA intercellular communication, but many studies have now shown that this process leads to gene expression changes in recipient cells. (81-83)

Circulating miRNAs can be associated with a variety of lipid-based vesicles and lipoproteins, as well as with lipid-free protein complexes. The physiological relevance of miRNA intercellular communication to wellness or disease remains to be determined; however, circulating miRNAs show great promise as novel disease biomarkers and may lead to new diagnostic tests. The excitement for miRNAs as biomarkers is mounting as more and more evidence supports that these noncoding RNAs are actively secreted from diseased tissues, possibly before the onset of overt disease. Circulating miRNAs may also represent a new way to intervene therapeutically in treating a wide variety of diseases and in better understanding gene regulation.(4)

miRNA and Lipoprotein Metabolism

The primary functions of lipoproteins are the delivery of neutral lipids, such as triglycerides, and to a lesser degree cholesterol to peripheral cells and the removal of excess

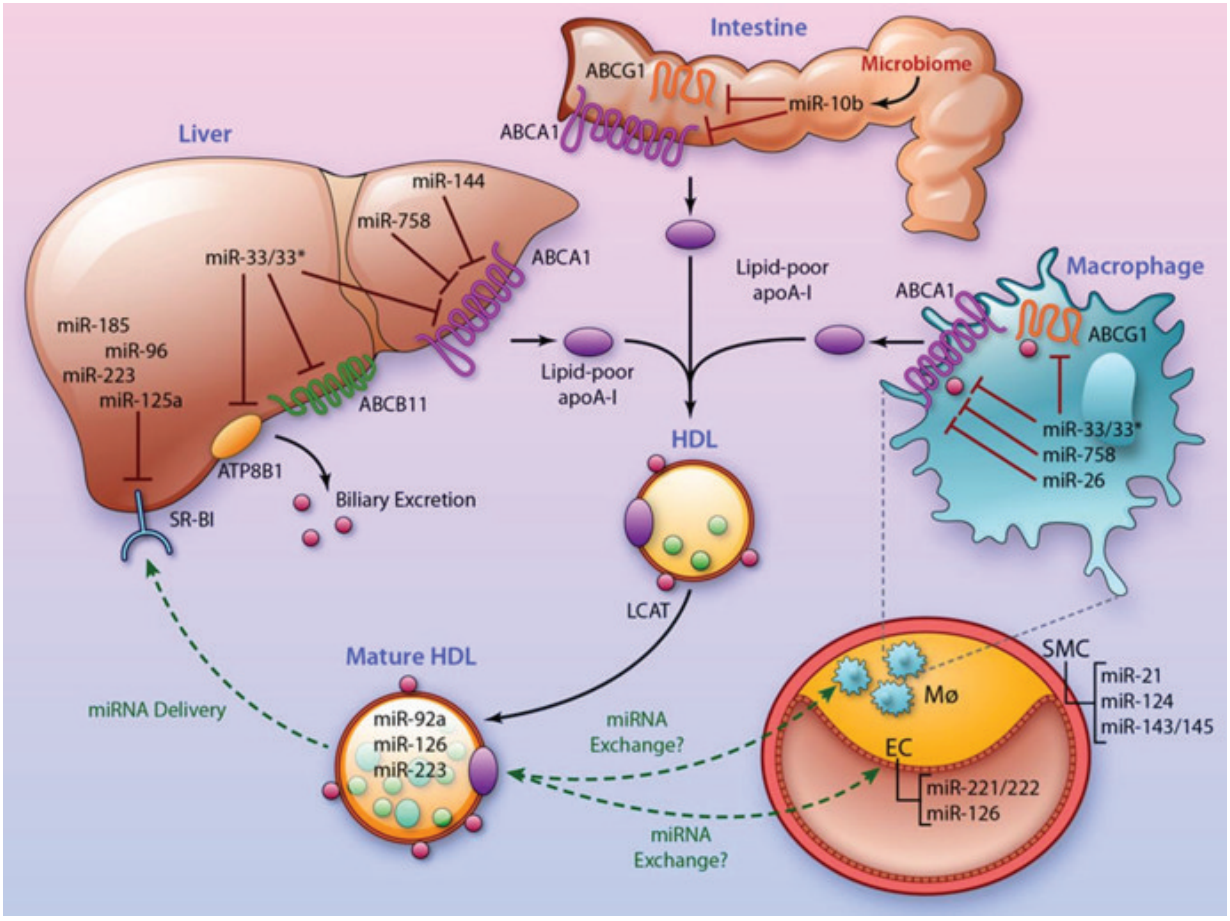

Figure 2. miRNA coordination of HDL homeostasis.(6) miRNAs have been shown to regulate genes involved in cholesterol efflux and HDL homeostasis in various tissues, including the liver, intestine, and macrophage (Adapted with permission from American Heart Association).

cellular cholesterol by the reverse cholesterol transport pathway.(84) The epicenter of lipoprotein metabolism resides in the liver, which has been the focus of many miRNA studies.(85-87) Insufficient or excessive cellular cholesterol results in pathologic processes including atherosclerosis and metabolic syndrome.(88-91) The mechanisms controlling this balance remain incompletely understood (92).

Many diseases result from perturbations in lipid homeostasis, including atherosclerosis, type II diabetes and metabolic syndrome.(88-90,93) The intracellular and membrane levels of fatty acids and cholesterol are under constant surveillance and are coordinated with *de novo* lipid biosynthesis by endoplasmic reticulum (ER)-bound sterol regulatory element-binding proteins (SREBPs).(94-96)

Lipid metabolism is tightly regulated at the cellular level. In addition to classic transcriptional regulation of cholesterol metabolism (*e.g.* by SREBP and liver X receptor (LXR)), miRNAs have now been identified to be potent post-transcriptional regulators of lipid metabolism genes involved in cholesterol homeostasis and fatty acid oxidation.(92) Recent discoveries of miRNAs that control high-density lipoprotein abundance and function have expanded our knowledge of the mechanisms regulating this important lipoprotein subclass. miRNAs have been shown to regulate gene networks that control high-density lipoprotein biogenesis and uptake, as well as discrete steps in the reverse cholesterol transport pathway.(6)

Nascent HDL is generated in the liver through the efflux of cholesterol and phospholipid across the hepatocyte membrane onto newly synthesized lipid-poor apolipoprotein A-I.3 The ATP-binding cassette transporter A1 (ABCA1) plays a critical role in this process as evidenced by the near-absence of plasma HDL-C in patients with Tangier disease, which results from mutations in the ABCA1 gene.(97-100) The levels of ABCA1 at the plasma membrane controls the rate of cholesterol efflux to apolipoprotein A-I, and hepatic-specific deletion of ABCA1 results in an ≈85% loss of total HDL-C, with ABCA1 in the adipose and intestine contributing to the residual balance.(101-105)

Several miRNAs have recently been identified that target ABCA1 and thus regulate plasma levels of HDL-C. Among these, miR-33a and miR-33b were the first to be reported (106,107) and were intriguing because of their genomic context: miR-33a and miR-33b are embedded in intronic regions of SREBP2 and SREBP1 genes that code for the SREBP2 and SREBP1 transcription factors that control the expression of genes involved in cholesterol and fatty acid synthesis. MiR-33a/miR-33b are coregulated with their host genes and act to repress gene programs that oppose SREBP functions.

The physiological relevance of miR-33 targeting of ABCA1 was initially demonstrated using inhibitors of miR-33, which increased cholesterol efflux from hepatocytes to apolipoprotein A-I *in vitro* and raised levels of plasma HDL-C in mice by 25% to 30%. These findings were subsequently confirmed by targeted deletion of miR-33, which resulted in 25% and 40% increases in plasma HDL-C in male and female miR-33 null mice, respectively (108).

Indeed, shortly after the discovery of miR-33, other miRNAs were found to repress ABCA1 and cholesterol efflux *in vitro*, including miR-758 (109), miR-26 (110) and miR-106b (111). Recently, 2 groups reported that miR-144, an intergenic miRNA present in a bicistronic cluster with miR-451, also targets ABCA1 in the liver and modulates plasma HDL-C levels.(112,113) Interestingly, miR-144 expression is regulated by 2 members of the nuclear hormone receptor family: the farsenoid X receptor and LXR (112,113), providing fine-tuning of ABCA1 expression under specific biological contexts.

Collectively, the studies of miR-33 and miR-144 have begun to illuminate the intricate network that fine-tunes ABCA1-dependent cholesterol efflux from the liver to regulate plasma HDL-C. It is likely that numerous other miRNAs will be identified to act in concert to regulate ABCA1 expression in the liver, with their individual and combined contributions determined by factors that regulate miRNA expression and abundance. The identification of such metabolic rheostats will no doubt provide new opportunities for therapeutic manipulation of plasma HDL-C levels and RCT. The efflux of excess cholesterol from peripheral tissues, particularly macrophages in the artery wall (102,104), is essential for maintaining cholesterol homeostasis. At the cellular level, this requires that cholesterol first be mobilized from internal stores via cooperation of the lysosome, lipid droplets, neutral cholesteryl ester hydrolase, and the autophagy machinery.(114) The final step of cholesterol efflux from the plasma membrane is mediated by ABCA1 to lipid-poor apolipoprotein A-I (ApoE in the brain) and through the related transporter, ABCG1, to mature HDL particles.

Autophagy, which regulates the availability of free cholesterol for efflux (115-118) and contributes prominently to macrophage RCT in vivo, is a complex process that requires multiple sequential membrane remodeling and trafficking events, orchestrated by a small army of autophagy-related gene (ATG) proteins. This pathway has recently been shown to be regulated by several miRNAs, including miR-18a, miR- 20a, miR-30a, miR-30d, miR-101, miR-106b, miR-132, miR-181a, miR-196, miR-212, miR-221, miR-222, miR-376b, miR-502 (119-127), which act by targeting ATG

proteins (ATG2, ATG4, ATG5 and ATG12) (14,113,114,127) or their upstream effectors (Beclin 1 (BECN1), mammalian target of rapamycin (mTOR) and uncoordinated 51-like kinase 1 (ULK1).(120,122,123,125,127)

Like miR-33, miR-26 also downregulates other genes involved in cholesterol mobilization in addition to ABCA1, such as ADP ribosylation factor-like 7, an intracellular transport protein that moves cholesterol to the membrane for removal by ABCA1.(110) Expression of miR-26 is suppressed by LXR and thus would be predicted to be downregulated under conditions of cholesterol excess during which increased levels of ABCA1 would be needed. However, miR-144 would be induced by LXR to target ABCA1 under similar conditions (112), and thus further studies of the temporal expression and abundance of these 2 miRNAs will be needed to resolve their relative contribution to cholesterol efflux control. Finally, miR-758 and miR-106b have been found to be highly enriched in the brain where ABCA1 plays a key role in effluxing excess cholesterol to ApoE, the predominant apolipoprotein in the brain (111,128).

Transport of HDL-C to the liver for bile acid synthesis and excretion is the final step of RCT and can occur either directly via SR-BI or after transfer to apolipoprotein B–containing lipoproteins by the CETP present in humans. miRNAs targeting these pathways are just beginning to be explored and represent exciting new therapeutic targets to influence the route of delivery of HDL's cargo (6).

Bioinformatic prediction algorithms, such as Targetscan and MiRanda, indicate that ≤50 miRNAs may target the 3′UTR of human SR-BI. Among these, miR-185, miR-96, and miR-223 were validated as strong repressors of SR-BI mRNA and cell surface expression, and their inhibition in HEPG2 cells increased SR-BI expression and selective HDL-C uptake.(129) In addition to its critical role in RCT, HDL can exert antiinflammatory, antioxidant, and antithrombotic effects, and these functions seem to vary among individuals. It is thus possible that these functions of HDL may be mediated in part by, or altered by, the subset of miRNAs that it carries. Indeed, the miRNA cargo of HDL has been shown to be altered in both mice and humans by hypercholesterolemia and atherosclerosis.(71)

miRNA-based therapeutics represent a new class of drugs that hold promise for the treatment of cardiovascular and other diseases. The recent Food and Drug Administration approval of Kynamro (previously known as Mipomersen), a first-inclass antisense oligonucleotide inhibitor that targets apolipoprotein B-100 to reduce LDL cholesterol for the treatment of homozygous familial hypercholesterolemia, represents a giant leap forward for oligonucleotide-based therapies, including miRNA therapeutics.(130) These results have generated considerable excitement for the possibility of using miRNA-based therapies to treat cardiovascular disorders. miR-33 inhibition is thought to be particularly promising as a therapeutic for atherosclerosis because it would enhance multiple components of the RCT pathway, including HDL biogenesis, cholesterol efflux from plaque macrophages, and cholesterol excretion to the bile. Indeed preclinical studies of miR-33 inhibition in mice and nonhuman primates for ≤12 weeks showed sustained increases in HDL-C (on the order of 40-50%).(12)

The prospect that HDL-carried miRNAs contribute to the heterogeneous effects of HDL on endothelial cells, macrophages, and other cell types that influence vascular health is intriguing and may provide insight into how the protective effects of HDL may be altered in disease or enhanced for therapeutic purposes (6).

miRNA and Atherosclerosis

Atherosclerosis is a multifactorial disease driven, in part, by chronic inflammation in response to cholesterol accumulation in the arterial wall.(131) The first major event in the progression of the early atheroma is the loss of endothelial integrity. Endothelium dysfunction facilitates the subendothelial accumulation of cholesterol-bearing lipoproteins, compromises vasodilation, and is both proinflammatory and prothrombotic.(132,133) A majority of what we understand about the role of miRNAs in endothelial cells comes from studies of angiogenesis. Endothelial migration studies, utilizing wound healing assays, revealed a significant role for let-7, miR-221, and miR-222 in endothelial function.(134-137) Furthermore, recent studies have shown that miR-92a prohibits angiogenesis, whereas miR-126 sustains vascular integrity and promotes angiogenic signaling.(138,139)

Atheroprotective stimuli induce communication between endothelial cells and smooth muscle cells (SMCs) through an miRNA- and extracellular-vesicle-mediated mechanism and that this may comprise a promising strategy to combat atherosclerosis.(140) Circulating endothelial progenitor cells (EPCs) have been demonstrated to play an integral role in endothelial integrity due to their ability to reinforce the endothelium with new healthy endothelial cells to replace damaged or apoptotic cells.(141,142) In a recent study, individuals with atherosclerosis, as defined by coronary artery disease (CAD), showed significantly higher expression of miR-221 and miR-222 in EPCs compared with non-CAD individuals.(143) Furthermore, miR-221/222

levels were observed to be inversely related to EPCs levels, as CAD individuals had significantly less EPCs numbers.

miR-126 was shown to provide an atheroprotective effect (144) and was reported to be upregulated by flow in a klf2-dependent manner in zebrafish embryos.(145) The shear-sensitive miR-19a contributes to the antiproliferative effect of shear flow (146), whereas miR-10a regulation is involved in the antiinflammatory properties of shear stress.(147) miRNAs also regulate SMCs functions, and in particular, the cluster comprising miR-143 and miR-145 has been described to be of crucial importance for proper SMCs function.(148-150)

Recent observations of miRNA profile changes in balloon-injury and carotid-ligation models have revealed dynamic flux of specific miRNAs in the arterial wall, as part of the larger proliferative response.(22,148) Specifically, miR-125a, miR-125b, miR-133, miR-143, miR-145, miR-365 appear to be downregulated, and miR-21, miR-146, miR-214, and miR-352 were observed to be upregulated in neointimal formation models.(22) Accordingly, these observations have led to loss of function knockdown experiments, demonstrating that miR-21 promotes proliferation and neointimal growth due to injury.(22,151)

The role of miRNAs in VSMCs proliferation has been extensively but not exhaustively investigated.(150,152-155) Recently, miR-145 has been demonstrated to be a key determinant in VSMCs differentiation and phenotype (156,157) and to be downregulated in both atherosclerosis and arterial-injury models.(22,148) Together with miR-145, miR-143 has recently been shown to regulate the VSMCs proliferative response to balloon-injury through alterations in cytoskeleton organization.(156) Moreover, miR-92a seemed to be highly specific for the atherosclerotic endothelium: macrophages and SMCs that were exposed to oxidized LDL (oxLDL) expressed miR-92a only at a low level.(157)

Loyer *et al*. have discovered an additional feature of miR-92, which is relevant to atherosclerosis: miR-92a expression is mediated by oxLDL. When endothelial cells were exposed to oxLDL and low shear stress, Loyer *et al*. found that they secreted interleukin-6 and monocyte chemoattractant protein-1, which could be reversed by inhibition of miR-92a. The expression of miR-92a in the endothelial cells of atherosclerosis prone areas seemed to be driven by signal transducer and activator of transcription 3 (STAT3).(158) These combined regulatory effects of miR-92a in endothelial activation make it thus an important regulator of atherosclerosis development.

The potential of miR-92a to be used both as a diagnostic and therapeutic target in atherosclerotic disease. Because this miRNA seems to particularly act on the endothelium in atherosclerosis-prone areas, both systemic and local therapeutic approaches using miRNA inhibitors could be of interest in both preventing atherosclerotic disease and minimizing tissue loss on infarction when atherosclerotic disease is irreversible.(157)

miRNA: Therapeutics and Diagnostics

miRNA profiling can provide additional information about the biological processes involved in atherosclerosis, but miRNAs have also been thought to be potential biomarkers and drug targets. Circulating miRNAs have many qualities that make them attractive candidate molecular biomarkers. They are stable and evolutionarily conserved, and the changes in their expression are often tissue or disease specific. They are found in many body fluids such as urine, plasma, serum, and cerebrospinal fluid.(159)

Prognostic and diagnostic value for circulating miRNAs in ischemic cardiac disease has been investigated in several up-to-date publications.(160-162) The use of high-sensitive realtime/quantivative PCR (Q-PCR) or array technique facilitates miRNAs detection in body fluids, even if miRNAs are present at very low concentration in the circulation. There are likely two classes of miRNA biomarkers: 1) those miRNAs secreted passively due to tissue stress, injury, or necrosis, and therefore may not reflect the biology associated with disease pathogenesis; and 2) those miRNAs actively and/or chronically secreted during disease progression and perhaps contribute to the pathogenesis. An example of the former comes from the identification of myocardium-derived miRNAs in the circulation following a myocardial infarction. miR-208 and miR-499 are muscle-specific miRNAs and were the first to be identified in the circulation of patients following an acute myocardial infarction (AMI) (163). De Rosa *et al.* (10) went on to show that in conjunction with miR-208 and miR-499, miR-133 is also found in the serum of patients suffering from an AMI, and that these miRNAs are indeed derived from the myocardium.

The implications of miRNAs in the pathological process of the cardiovascular system have been recognized recently, and research on miRNAs in relation to cardiovascular disease has become a rapidly evolving field.(108,164) It is known that miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, and miR-499 are muscle-specific miRNAs. Among them, miR-133b and miR-206 are expressed only in

skeletal muscle and miR-208a is expressed only in cardiac muscle.(165) Much like cardiac troponin (cTn) levels, cTnT and cTnI, which are reflective of cardiac muscle damage yet are a robust and sensitive measurement of an AMI, miR-133/208/499 are a result of tissue damage yet signal that a pathological event has taken place.(166) Similarly, miR-122 is a highly abundant miRNA in the liver, and it was recently reported that levels of miR-122 in the circulation correlated with disease severity in patients suffering from chronic hepatitis C.(167) And finally, hypercholesterolemia and nonalcoholic fatty liver disease (NAFLD) have both demonstrated unique circulating miRNA profiles, with both pathologies associated with elevated levels of the liver-specific miR-122, suggesting once again that this tissue-specific release may be a signal of injury rather than a mediator of pathology (167,168).

In a very recent study using 820 participants of the Bruneck study, taking into account prospective values of miR-126, miR-197 and miR-223 led to a reclassification for myocardial infarction in addition to the Framingham Risk Score.(161) Thus, these results suggest that circulating miRNAs can be released from the myocardium in association with cellular damage, and serum miR-133a can be used as a biomarker for myocardial injury.(165) In more chronic conditions, such as type 2 diabetes, atherosclerosis, and hypercholesterolemia, miRNAs have also been used as biomarkers, and may reflect an involvement in disease pathogenesis. miR-126 is released in apoptotic bodies into the circulation (144), and a decrease in miR-126 expression in the circulation results in loss of vascular integrity and impaired angiogenesis.(169,170)

Furthermore, miR-126 in the circulation is also reduced in patients with coronary atherosclerosis (171) and is inversely correlated with patients with high LDL levels (172), underscoring its potential importance in maintaining vascular homeostasis across multiple tissues. Intriguingly, in patients with overt diabetes, there were measurable alterations in miRNA expression in the circulation before the onset of disease, namely in miR-126, miR-223, and miR-15a.(170)

The field of miRNAs as biomarkers is in its infancy, and as such, many of these outstanding issues are slowly being resolved. Investigators are now going to greater lengths when preparing samples and analyzing miRNA content in plasma, and streamlined protocols and procedures are assisting in the comparison across data sets.(173) Recent advances in the understanding of lipid metabolism have revealed that miR-33, an intronic miRNA located within the SREBP2 gene, suppresses expression of the cholesterol transporter ABCA1 and lowers HDL levels. Conversely, mechanisms that inhibit miR-33 increase ABCA1 and circulating HDL levels, suggesting that antagonism of miR-33 may be atheroprotective.(174). Studies establish that raising HDL levels by anti-miR33 oligonucleotide treatment promotes reverse cholesterol transport and atherosclerosis regression and suggest that it may be a promising strategy to treat atherosclerotic vascular disease. As miRNA in atherosclerosis is still a relatively new field of research, the full clinical potential of these small RNAs in the diagnostics and treatment of CVDs remains to be elucidated.

Conclusion

It is clear that the potential of extracellular miRNA for use as both a diagnostic and a therapeutic tool is tremendous, and in the span of a few years, the fi eld has turned from skepticism to acceptance that these tiny nucleic acids might be playing a role in the extracellular space. Undoubtedly an improved understanding of miRNA export and uptake will aid in the therapeutic arena, and will greatly assist the tailoring of individual miRNA therapies for specific diseases and tissues and hopefully aid in the eradication of possibly preventable diseases like dyslipidemia and atherosclerosis.

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